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Methotrexate Inhibits Proteolysis of Dihydrofolate Reductase by the N-end Rule Pathway*

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The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue. In eukaryotes, the N-end rule pathway is a ubiquitin-dependent, proteasome-based system that targets and processively degrades proteins bearing certain N-terminal residues. Arg-DHFR, a modified dihydrofolate reductase bearing an N-terminal arginine (destabilizing residue in the N-end rule), is short lived in ATP-supplemented reticulocyte extract. It is shown here that methotrexate, which is a folic acid analog and high affinity ligand of DHFR, inhibits the degradation but not ubiquitination of Arg-DHFR by the N-end rule pathway. The degradation of other N-end rule substrates is not affected by methotrexate. We discuss implications of these results for the mechanism of proteasome-mediated protein degradation.

The surface of a protein molecule bears a number of peptide bonds, which are potential cleavage sites for proteases. Nonetheless, only some intracellular proteins are short-lived *in vivo*, indicating that most of these solvent-exposed bonds cannot be cleaved by intracellular proteases. Features of a protein that make it short-lived *in vivo* or *in vitro* are called degradation signals or degrons (Varshavsky, 1991). The resistance of a long-lived protein to proteases located in the same compartment is due in part to the sequence selectivity of proteases, which require the presence of a sequence motif or at least a specific residue in a substrate. In addition, the rate of peptide bond cleavage by even a relatively nonspecific protease depends on conformational flexibility of the motif that the protease recognizes. For example, only some of the potential cleavage sites on the surface of a globular protein are cleaved efficiently by the bacterial metalloendoprotease thermolysin, and these preferred cleavage sites are located in exposed segments of the polypeptide chain which have the highest spatial mobility (Fontana *et al.*, 1986).

Thus, even if a motif recognized by a protease is present on the surface of a protein, conformational rigidity of this potential degron may preclude its efficient utilization by the protease. Conversely, a conformationally destabilized protein may acquire degrons that are masked in an unperturbed version of the protein (Parsell and Sauer, 1989). This can happen not only through conformational relaxation of a previously rigid (and therefore cryptic) surface degron but also through exposure of degrons in previously buried regions of the protein. The mechanistic connection between segmental mobility of a polypeptide chain and its susceptibility to proteolysis stems from a scarcity of local chain conformations that can lend themselves to an optimal transition-state intermediate without a conformational adjustment (Fontana *et al.*, 1986; Creighton, 1992; Hubbard *et al.*, 1994).

Dynamic aspects of a substrate's conformation are likely to play a major role in the functioning of intracellular proteolytic systems such as those that involve the multisubunit, multicatalytic protease called the proteasome (for reviews, see Lupas *et al.*, 1995; Rechsteiner *et al.*, 1993; Goldberg and Rock, 1992; Orlowski, 1990). A salient feature of these ATP-dependent systems is their processivity: once the degradation of a protein begins, it proceeds to completion. Thus, a proteasome-mediated system should be able to perturb the conformation of a globular protein substrate before or during its processive degradation by the proteasome. The "conformational" problem to be solved by the proteasome is analogous to the problem faced by a protein translocation system: components of a transmembrane channel must unfold a protein before or during its "threading" across membrane, except that in this case the protein is transported rather than destroyed (Blobel, 1980; Eilers and Schatz, 1986; Sanders and Schekman, 1992; Arkowitz *et al.*, 1992).

In the present work, we show that a proteasome-based proteolytic system called the N-end rule pathway is remarkably sensitive to alterations in the conformational stability of its substrates. The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (for review see Varshavsky, 1992). Similar but distinct versions of the N-end rule operate in all organisms examined, from mammals to bacteria (Bachmair *et al.*, 1986; Gonda *et al.*, 1989; Tobias *et al.*, 1991). In eukaryotes, the N-end rule-based degradation signal, called the N-degron, comprises two determinants: a destabilizing N-terminal residue and an internal lysine (or lysines) of a substrate (Bachmair and Varshavsky, 1989; Johnson *et al.*, 1990; Hill *et al.*, 1993). The Lys residue is the site of formation of a multiubiquitin chain (Chau *et al.*, 1989). Ubiquitin (Ub)[1] is a protein whose covalent conjugation to other proteins (often in the form of a multi-Ub chain) plays a role in a number of processes, primarily through routes that involve protein degradation (for reviews, see Ciechanover and Schwartz, 1994; Parsell and Lindquist, 1993; Vierstra, 1993; Jentsch, 1992; Varshavsky, 1992; Finley, 1992; Hochstrasser, 1992).

The recognition of an N-end rule substrate is mediated by a

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[1] The abbreviations used are: Ub, ubiquitin; MTX, methotrexate; DHFR, mouse dihydrofolate reductase; ha, epitope tag derived from hemagglutinin; Tricine, *N*-[2-hydroxyl-1,1-bis(hydroxymethyl)-ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; β-gal, *E. coli* β-galactosidase.

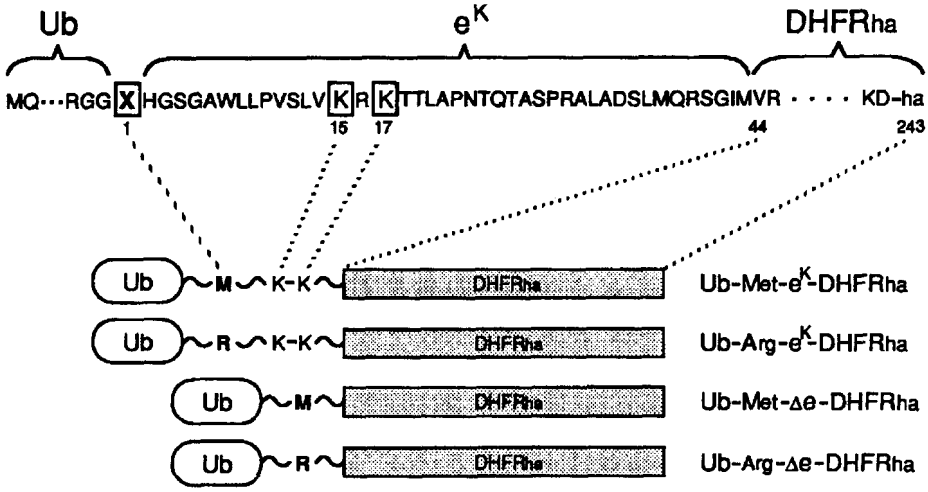

FIG. 1. **Test proteins.** The proteins shown (Ub-Met-e^K-DHFRha, Ub-Arg-e^K-DHFRha, Ub-Met-Δe-DHFRha, and Ub-Arg-Δe-DHFRha) were expressed in *E. coli*, purified, and used as test substrates in ATP-supplemented reticulocyte extract (see "Materials and Methods"). DHFRha is DHFR whose C terminus was extended with a 14-residue sequence containing the hemagglutinin (ha) epitope (see "Materials and Methods"). The 43-residue region (derived from *E. coli* Lac repressor) between the Ub and DHFRha moieties in Ub-Met-e^K-DHFRha and Ub-Arg-e^K-DHFRha is denoted by e^K (Bachmair and Varshavsky, 1989; Johnson *et al.*, 1992). The residues are numbered from a residue *X* at the Ub-e^K junction to the last residue of the ha epitope in Ub-*X*-e^K-DHFRha. The sequence of DHFR-linked e^K in the present work differs from that of the βgal-linked e^K near the e^K-reporter junction (Johnson *et al.*, 1992). Lys-15 and Lys-17 residues are *boxed* in the sequence of e^K. In Ub-Met-Δe-DHFRha and Ub-Arg-Δe-DHFRha, e^K was replaced by the sequence Met/Arg-His-Gly-Ser-Gly-Ile-Met between Ub and Val, the wild-type N-terminal residue of mouse DHFR (Dohmen *et al.*, 1994).

protein called N-recognin or *E*3. The binding of N-recognin to a substrate's destabilizing N-terminal residue is followed by the formation of a multi-Ub chain linked to an internal lysine of the substrate (the second determinant of its N-degron). A substrate-linked multi-Ub chain is required for the degradation of at least some N-end rule substrates (Chau *et al.*, 1989; Dohmen *et al.*, 1991). In both yeast and mammals, the ~200 kDa N-recognin and a specific ~20-kDa Ub-conjugating (*E*2) enzyme (one of several such enzymes in a cell) are physically associated, forming a part of a larger targeting complex (Madura *et al.*, 1993; Hershko and Ciechanover, 1992). A substrate bearing a multi-Ub chain is transferred (presumably while still bound by the targeting complex) to the 26 S proteasome, a ~2,000-kDa multicatalytic protease that contains about 40 distinct subunits (Rechsteiner *et al.*, 1993). The ensuing processive degradation of the substrate yields short (~10-residue) peptides and regenerates Ub from a multi-Ub chain. All Ub-dependent proteolytic systems, including the N-end rule pathway, apparently share many components of the 26S proteasome. Differences among these pathways include their distinct targeting complexes, whose recognins (associated with specific *E*2s) bind to degradation signals other than N-degrons (Varshavsky, 1992).

Previous studies (Gonda *et al.*, 1989; Reiss *et al.*, 1988) showed that the N-end rule pathway is active in ATP-supplemented extract from rabbit reticulocytes. In the present work we used this *in vitro* system and methotrexate (MTX; a folic acid analog and inhibitor of the enzyme dihydrofolate reductase (DHFR)), to determine whether noncovalent conformational stabilization of a protein affects its degradation by the N-end rule pathway.

MATERIALS AND METHODS

DNA Constructs—The plasmids pEJJ1-R and pEJJ1-M expressed, respectively, Ub-Arg-e^K-DHFRha and Ub-Met-e^K-DHFRha (Fig. 1) from the T7 polymerase promoter in *Escherichia coli* and were constructed using a three-part ligation as follows. (i) The pT7-7 vector (Tabor and Richardson, 1985) was digested with *Nde*I and *Hin*dIII, and the larger fragment was purified by agarose gel electrophoresis, yielding the first of three fragments to be ligated. (ii) A fragment encoding the Ub-Arg/Met portions of the above fusions was produced from the plasmid pLG-Ub-M-DHFR (Bachmair and Varshavsky, 1989) using polymerase chain reaction (Ausubel *et al.*, 1992) and the primer 5′-GAATTC-CATATGCAGATTTTCGTGAAGAC-3′ (the underlined sequence is the *Nde*I site, whose ATG is the start codon of the *UBI4* gene (Özkaynak *et al.*, 1987)). The 3′-primer for this polymerase chain reaction encompassed the *Bgl*II site near the junction between sequences encoding the e^K extension (Fig. 1) and DHFR. (iii) The third fragment to be ligated, encoding e^K-DHFRha, was a ~600-base pair *Bam*HI-*Hin*dIII fragment derived from pLG-Ub-M-DHFRha (Johnson, 1992). Ligation of these three fragments (the second one encoding either Arg or Met at the Ub-e^K junction) yielded pEJJ1-R and pEJJ1-M, encoding, respectively, Ub-Arg-e^K-DHFRha and Ub-Met-e^K-DHFRha. The plasmids pPJJ1-R and pPJJ1-M, expressing, respectively, Ub-Arg-Δe-DHFRha and Ub-Met-Δe-DHFRha (Fig. 1), were produced from pEJJ1-R and pEJJ1-M by excising their *Bam*HI-*Hin*dIII fragment (encoding e^K-DHFRha) and replacing it with a ~600-base pair fragment of pPW17-R (Dohmen *et al.*, 1994), which encoded Arg-His-Gly-Ser-Gly-Ile-Met-DHFRha.

E. coli JM101 and DH5α (Ausubel *et al.*, 1992) were used as hosts in plasmid construction and in plasmid preparation for sequencing, respectively. The final constructs were verified by restriction mapping and nucleotide sequencing (Ausubel *et al.*, 1992). *E. coli* BL21(DE3) (Studier and Moffat, 1986) was used for overexpression of DHFR-containing fusions.

Ub-X-DHFRha Test Proteins—Unless stated otherwise, all procedures were carried out at 4 °C. 0.5 ml of an overnight culture of *E. coli* BL21(DE3) carrying one of the pT7-based plasmids was diluted into 50 ml of Luria broth containing ampicillin at 75 μg/ml, and the culture was grown at 37 °C to an A_{600} of ~0.2; more ampicillin was then added, to a final concentration of 115 μg/ml, and the incubation was continued until an A_{600} of ~0.6. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.2 mM. The culture was grown for another 25 min at 30 °C. The cells were harvested by centrifugation at 4,000 × *g* for 10 min; washed twice with M9 buffer (Ausubel *et al.*, 1992); resuspended in 1 ml of M9 supplemented with 0.22% glucose, rifampicin (0.2 mg/ml), ampicllin (75 μg/ml), and 0.1% methionine assay medium (Difco); and incubated with 0.5 mCi of Tran^{35}S-label (ICN) for 10 min at 30 °C. The cells were collected by centrifugation, washed once with Luria broth, twice with M9 buffer, and resuspended in 0.5 ml of 25% (w/v) sucrose, 50 mM Tris-HCl, pH 8.0. 0.1 ml of a lysozyme solution (10 mg/ml in 0.25 M Tris-HCl, pH 8.0) was then added. After 5 min at room temperature, 0.8 ml of the lysis buffer (1% Triton X-100, 37 mM Na-EDTA, 50 mM Tris-HCl, pH 8.0) was added, and the suspension was vortexed for 10 s twice, followed by a quick freezing in liquid N_2 and thawing of the sample in a 37 °C bath. The lysate was centrifuged at 40,000 × *g* for 25 min, and Ub-*X*-DHFRha (this term denotes both Ub-*X*-e^K-DHFRha and Ub-*X*-Δe-DHFRha; Fig. 1) in the supernatant was bound to the monoclonal anti-ha antibody 12CA5 (Field *et al.*, 1988; Johnson *et al.*, 1992), added as an ascitic fluid,

in a roughly 100-fold molar excess over Ub-*X*-DHFRha. The antibody-bound Ub-*X*-DHFRha was immobilized on a 0.5-ml column of protein A-Sepharose (Repligen, Cambridge, MA). The column was washed with 5 ml of 10 mM sodium phosphate buffer, pH 7.5. Ub-*X*-DHFRha was eluted with 2.5 M LiCl in the same buffer. The sample was dialyzed overnight against 50 mM Tris-HCl, pH 7.5, then concentrated by dialysis for ~20 h against 50% (v/v) glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, and stored at −20 °C. The specific radioactivity of ^{35}S-labeled Ub-*X*-DHFRha was ~10^4 cpm/μg. Protein concentrations were determined using the Bradford (Coomassie Blue) assay (Pierce). Ub-DHFR fusions purified as described above were conformationally unperturbed in that they could be retained on an MTX-Sepharose column (Pierce).

In Vitro Assays with Reticulocyte Extract—Rabbit reticulocytes were purchased from Green Hectares (Oregon, WI) and shipped overnight on ice. ATP-depleted reticulocyte extract was prepared as described by Gonda *et al.* (1989) and stored as 0.2-ml samples in liquid N_2. Only once-frozen samples were used in all experiments. An assay mixture contained 75 μl of the extract, 10 μl of an ^{35}S-labeled Ub-*X*-DHFRha, 0.1 mM dithiothreitol, 5 mM $MgCl_2$, 0.8 mM ATP, and an ATP-regenerating system (10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase). Assays were performed as follows. A reaction mixture complete except for ATP and the ATP-regenerating system was incubated for 10 min at 37 °C to allow for the deubiquitination of a Ub-*X*-DHFRha fusion protein; an aliquot was then withdrawn (time zero in Figs. 2 and 3), ATP and an ATP-regenerating system were added to start ATP-dependent reactions in the extract, and the incubation continued at 37 °C.

The initial concentration of an ^{35}S-labeled Ub-*X*-DHFRha in the extract was ~5 μg/ml; control experiments (not shown) indicated that this concentration was not rate-limiting for the degradation of either Arg-e^K-DHFRha or Arg-Δe-DHFRha. MTX (where indicated) was added from a 2 M stock solution in 0.2 M NaOH to Ub-*X*-DHFRha in a reaction tube and incubated for 5 min at 0 °C before assembling the reaction mixture as described above. The final MTX concentration was 20 μM. Two equal samples were withdrawn from reaction tubes at the times indicated in the legends to Figs. 2 and 3. One sample was assayed for the amount of 5% trichloroacetic acid-soluble ^{35}S, and the other was examined by a Tricine buffer-based SDS-PAGE (9% acrylamide, 0.24% bisacrylamide) (Schägger and Jagow, 1987). The gels were analyzed using fluorography or PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Trichloroacetic acid-soluble ^{35}S was determined as follows. 0.1 ml of a solution of bovine serum albumin (50 mg/ml) was added to a 10-μl sample; 0.11 ml of 10% trichloroacetic acid was then added; the sample was incubated on ice for 10 min, followed by centrifugation at 12,000 × *g* for 5 min. ^{35}S in the supernatant was determined using a water-compatible scintillation mixture and a scintillation counter. In each of the graphs of Fig. 3, the amount of trichloroacetic acid-soluble ^{35}S in a zero-point sample (before the addition of ATP; 5–10% of total ^{35}S) was subtracted from the values for samples withdrawn at later times.

RESULTS

Linear Ub fusions are rapidly cleaved *in vivo* or in cell-free extracts after the last residue of Ub, making possible the production of otherwise identical proteins bearing different N-terminal residues (Bachmair *et al.*, 1986; Baker *et al.*, 1992). In one application of this method, Gonda *et al.* (1989) incubated fusions of Ub to *E. coli* β-galactosidase (Ub-*X*-βgal) in reticulocyte extract, producing *X*-βgal test proteins bearing different N-terminal residues. Depending on the identity of a residue *X*, an *X*-βgal is either short-lived or metabolically stable in ATP-supplemented extract (Gonda *et al.*, 1989), similar to the *in vivo* findings with the same *X*-βgals in the yeast *Saccharomyces cerevisiae* (Bachmair *et al.*, 1986). Mutational analysis has shown that either one of two lysines (Lys-15 or Lys-17) in a non-βgal N-terminal region of an *X*-βgal test protein was also required for rapid degradation of *X*-βgal by the N-end rule pathway (Fig. 1) (Bachmair and Varshavsky, 1989; Johnson *et al.*, 1990). The function of these lysines was revealed by the finding that Lys-15 or, alternatively, Lys-17, is the site of formation of a multi-Ub chain (Chau *et al.*, 1989). The non-βgal, ~40-residue extension at the N terminus of βgal was derived in part from an internal sequence of *E. coli* Lac repressor (Bachmair and Varshavsky, 1989).

In the present work, the strategy used by Gonda *et al.* (1989) with βgal-based substrates was employed to examine the degradation of similarly designed DHFR-based substrates by the N-end rule pathway in reticulocyte extract. The ~40-residue N-terminal extension of DHFR, derived from *X*-βgal test proteins (Fig. 1) (Bachmair and Varshavsky, 1989), is denoted below as e^K (extension (e) containing lysine (K)) (Johnson *et al.*, 1992). In the constructs of Fig. 1, the C terminus of Ub-*X*-e^K-DHFR was extended with a 14-residue sequence containing ha, an epitope tag derived from the influenza virus hemagglutinin, which could be recognized by a monoclonal antibody (Field *et al.*, 1988; Johnson *et al.*, 1992). Two fusion proteins, Ub-Met-e^K-DHFRha and Ub-Arg-e^K-DHFRha (Fig. 1), bore, respectively, Met and Arg, a stabilizing and a destabilizing residue at the junction between Ub and the rest of a fusion (Varshavsky, 1992). These proteins were overexpressed in *E. coli*, labeled *in vivo* with [^{35}S]methionine, and purified by affinity chromatography, using anti-ha antibody (see "Materials and Methods").

Ub-Met-e^K-DHFRha and Ub-Arg-e^K-DHFRha were rapidly deubiquitinated upon addition to ATP-depleted reticulocyte extract, yielding, respectively, Met-e^K-DHFRha and Arg-e^K-DHFRha (Fig. 2, *A* and *B*, *lanes a* and *b*), analogous to the previously characterized deubiquitination of Ub-*X*-e^K-βgal fusions under the same conditions (Gonda *et al.*, 1989). Both Met-e^K-DHFRha and Arg-e^K-DHFRha remained metabolically stable in ATP-depleted extract (data not shown), but the addition of ATP resulted in a much faster degradation of Arg-e^K-DHFRha than Met-e^K-DHFRha, which remained long lived in ATP-supplemented extract (Fig. 2, *A* and *B*, *lanes b–e*, and Fig. 3*A*). The metabolic fates of DHFR-based test proteins were monitored by SDS-PAGE (Fig. 2) and also by measuring the amount of acid-soluble ^{35}S released during the incubation of an ^{35}S-labeled protein in ATP-supplemented extract (Fig. 3).

As shown in Fig. 3*A*, 33% of the initially present ^{35}S-labeled Arg-e^K-DHFRha was degraded to acid-soluble fragments in ATP-supplemented reticulocyte extract after a 30-min incubation. By contrast, only 6% of the otherwise identical Met-e^K-DHFRha, bearing an N-terminal Met (stabilizing residue in the N-end rule), was degraded after a 30-min incubation (Fig. 3*A*). Analysis by SDS-PAGE showed a transient accumulation of multiply ubiquitinated Arg-e^K-DHFRha derivatives and a decrease in intensity of the band of unmodified Arg-e^K-DHFRha in the course of its incubation in ATP-supplemented extract (Fig. 2*B*). By contrast, Met-e^K-DHFRha was neither ubiquitinated nor significantly degraded in ATP-supplemented extract (Fig. 2*A*), in agreement with the findings about similarly designed *X*-βgal substrates (Gonda *et al.*, 1989).

Previous work has shown that an amino acid derivative such as a dipeptide that bears a destabilizing N-terminal residue can inhibit the degradation of a βgal-based N-end rule substrate either *in vitro* (reticulocyte extract) (Gonda *et al.*, 1989) or *in vivo* (yeast cells) (Baker and Varshavsky, 1991). As shown in Figs. 2*C* and 3*A*, the degradation of a DHFR-based N-end rule substrate such as Arg-e^K-DHFRha in reticulocyte extract was almost completely inhibited by the Arg-Ala dipeptide. Dipeptides bearing destabilizing N-terminal residues inhibit the N-end rule pathway by competing with N-end rule substrates for binding to N-recognin (Reiss *et al.*, 1988; Gonda *et al.*, 1989; Baker and Varshavsky, 1991). Arg-Ala not only precluded the degradation of Arg-e^K-DHFRha (Fig. 3*A*) but also inhibited its ubiquitination (Fig. 2*C*), indicating that Arg-Ala blocks a step in the N-end rule pathway which precedes the ubiquitination step.

We asked whether the folate analog MTX, a high affinity DHFR ligand (K_d of ~10^{-11} M) and a competitive inhibitor of DHFR (Matthews *et al.*, 1985), would affect the degradation of

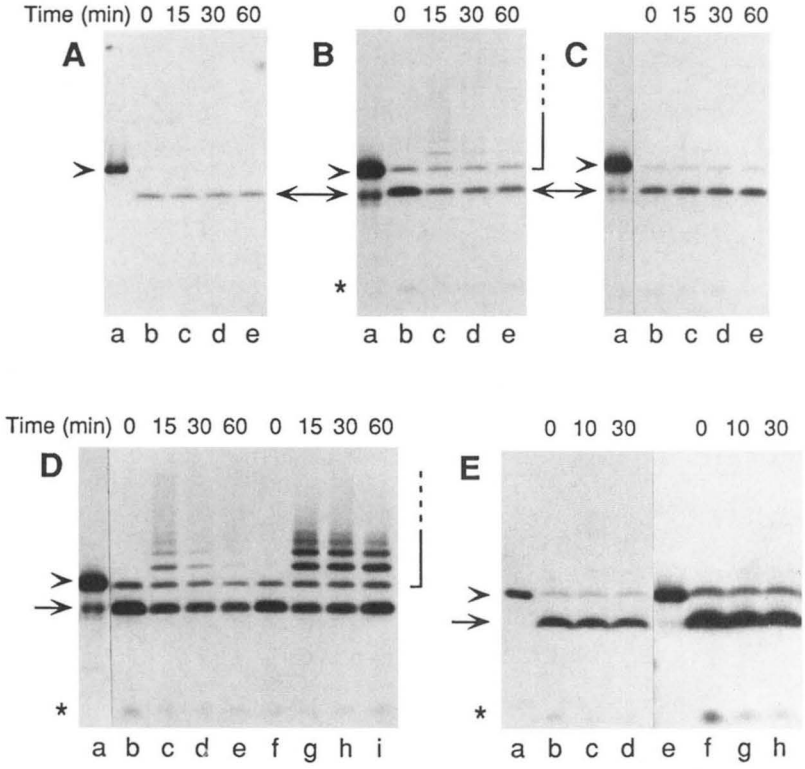

FIG. 2. **Effect of MTX on the degradation of DHFR-based N-end rule substrates in reticulocyte extract.** *Panel A*: *lane a*, purified, ^{35}S-labeled Ub-Met-e^{K}-DHFRha (the amount of substrate added to this lane and analogous lanes in other panels was larger than the initial amounts of substrate in *lane b* and analogous lanes). *Lane b*, Ub-Met-e^{K}-DHFRha was added to ATP-depleted reticulocyte extract and incubated for 10 min at 37 °C (time zero sample). *Lanes c–e*, same as *lane a*, but the samples were withdrawn and analyzed by SDS-PAGE 15, 30, and 60 min after the addition of ATP at time zero (see "Materials and Methods"). *Panel B*: same as *panel A* but with Ub-Arg-e^{K}-DHFRha. *Panel C*: same as *panel B*, but the Arg-Ala dipeptide was added to reticulocyte extract (to the final concentration of 10 mM) together with Ub-Arg-e^{K}-DHFRha. *Panel D*: *lanes a–e*, same as *panel B*, but the data are from another experiment. *Lanes f–i*, same as *lanes b–e*, but the assay was carried out in the presence of 20 μM MTX (see "Materials and Methods"). *Panel E*: *lane a*, purified, ^{35}S-labeled Ub-Arg-Δe-DHFRha. *Lanes b–d*, same as *lanes b–d* in *panel A*, but the assay was carried out with Ub-Arg-Δe-DHFRha, and the incubation times were 0, 10, and 30 min (after the addition of ATP). *Lanes e–h*, same as *lanes a–d*, but the assay was carried out in the presence of 20 μM MTX. *Arrowheads* and *arrows* indicate, respectively, the bands of 36-kDa Ub-*X*-e^{K}-DHFRha fusions and their 28-kDa deubiquitinated derivatives, *X*-e^{K}-DHFRha (Ub-Arg-Δe-DHFRha and Arg-Δe-DHFRha in *panel E*). *Asterisks* indicate the band of 8-kDa Ub, produced by deubiquitination of ^{35}S-labeled Ub-*X*-e^{K}-DHFRha and Ub-Arg-Δe-DHFRha. *Half-open square brackets* denote the bands of multiubiquitinated Arg-e^{K}-DHFRha. The incubation times are indicated above the *lanes*.

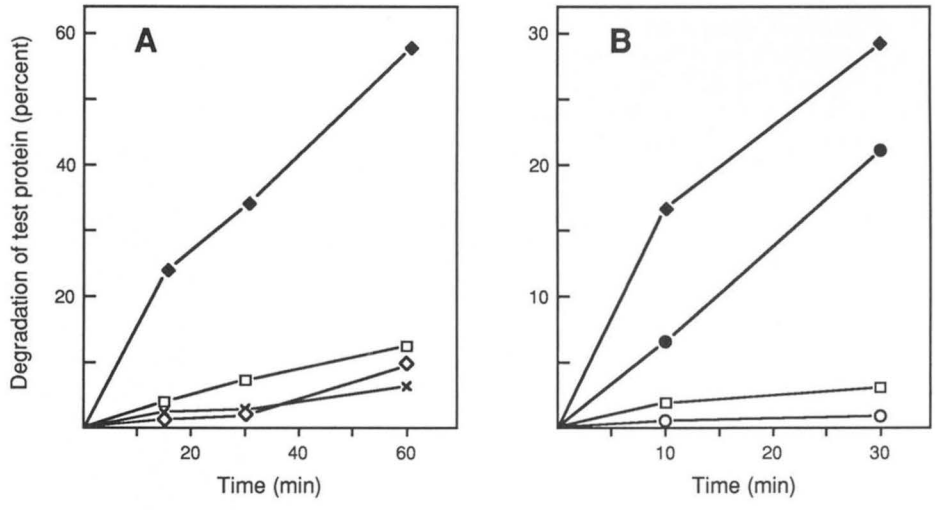

FIG. 3. **Degradation of DHFR-based N-end rule substrates in reticulocyte extract, measured by determining acid-soluble ^{35}S.** *Panel A*: □, Met-e^{K}-DHFRha; ♦, Arg-e^{K}-DHFRha; ×, Arg-e^{K}-DHFRha in the presence of 1 mM Arg-Ala dipeptide; ◇, Arg-e^{K}-DHFRha in the presence of 20 μM MTX. *Panel B*: □, same as in *panel A* but from another experiment with Met-e^{K}-DHFRha; ♦, Arg-e^{K}-DHFRha; ●, Arg-Δe-DHFRha; ○, Arg-Δe-DHFRha in the presence of 20 μM MTX. Each decay curve was determined at least three times, in independent experiments, with the results differing by less than 15% for each of the time points. See "Materials and Methods" for the definition of a zero time point and other details.

a DHFR-based N-end rule substrate. Remarkably, the presence of MTX in ATP-supplemented reticulocyte extract resulted in a nearly complete inhibition of Arg-e^{K}-DHFRha degradation (Fig. 3*A*). The inhibitory effect of MTX was confined to the actual proteolysis of Arg-e^{K}-DHFRha: its multiubiquitination was in fact enhanced by MTX, in contrast to the effect of Arg-Ala, which inhibited both the degradation and ubiquitination of Arg-e^{K}-DHFRha (Fig. 2*D*; compare with Fig. 2*C*).

We also determined the effect of MTX on degradation of Arg-Δe-DHFRha (derived from Ub-Arg-Δe-DHFRha), which lacked most of the ~40-residue, lysine-containing e^{K} extension of Arg-e^{K}-DHFRha (Fig. 1). Previous work has shown that Arg-Δe-DHFRha is much longer lived than Arg-e^{K}-DHFRha in the yeast *S. cerevisiae* at 30 °C ($t_{1/2}$ of more than 4 h *versus* ~10 min, respectively); it has also been shown that a major reason for the increased metabolic stability of Arg-Δe-DHFRha is the

absence of Lys-15 and Lys-17 residues: Arg-$e^{\Delta K}$-DHFRha, which contains Arg instead of Lys at positions 15 and 17 of the otherwise unaltered e^K extension, is nearly as long-lived in yeast as Arg-Δe-DHFRha (Bachmair and Varshavsky, 1989). In a qualitative agreement with these *in vivo* data, Arg-Δe-DHFRha was degraded more slowly than Arg-e^K-DHFRha in ATP-supplemented reticulocyte extract (Fig. 3*B*). The degradation of Arg-Δe-DHFRha was mediated by the N-end pathway, inasmuch as Met-Δe-DHFRha, bearing a stabilizing N-terminal residue, was degraded at a much lower rate than Arg-Δe-DHFRha (data not shown).

Similarly to the findings with Arg-e^K-DHFRha (Figs. 2*D* and 3*A*), the addition of MTX almost completely inhibited the degradation of Arg-Δe-DHFRha in ATP-supplemented reticulocyte extract (Fig. 3*B*). In contrast to the extensive ubiquitination of Arg-e^K-DHFRha prior to its degradation (Fig. 2, *B* and *D*), the degradation of Arg-Δe-DHFRha was not accompanied by a significant accumulation of its multiubiquitinated derivatives, and no enhancement of multiubiquitination of Arg-Δe-DHFRha could be detected in the presence of MTX as well (Fig. 2*E*).

The effect of MTX was confined to DHFR-based substrates: in parallel assays with βgal-based N-end rule substrates such as Arg-e^K-βgal (Gonda *et al.*, 1989), the addition of MTX did not alter the kinetics of Arg-e^K-βgal degradation (data not shown).

DISCUSSION

The MTX-DHFR assay has been used previously to address the mechanistic and kinetic aspects of protein translocation across membranes (Eilers and Schatz, 1986, 1988; Vestweber and Schatz, 1988; Wienhues *et al.*, 1991; Arkowitz *et al.*, 1992). The present work extends the applications of MTX-DHFR to the problem of Ub-dependent protein degradation. We report the following results.

1) Arg-e^K-DHFRha (Fig. 1) is degraded by the N-end rule pathway in ATP-supplemented reticulocyte extract in the absence but not in the presence of MTX, a folic acid analog and a competitive inhibitor of DHFR which binds to mammalian DHFRs with a K_d of $\sim 10^{-11}$ M (Fig. 3*A*).

2) The effect of MTX is confined to DHFR-based substrates, in that the degradation of other N-end rule substrates such as Arg-e^K-βgal is not inhibited by MTX.

3) The degradation of Arg-e^K-DHFRha (this work) and other N-end rule substrates (Gonda *et al.*, 1989) in reticulocyte extract is highly processive: no degradation intermediates could be detected by SDS-PAGE in the course of proteolysis.

4) MTX inhibits the degradation but not ubiquitination of Arg-e^K-DHFRha, whose multiubiquitinated derivatives become more abundant in the presence of MTX (Fig. 2*D*). Previous work (Chau *et al.*, 1989; Bachmair and Varshavsky, 1989) has shown that the degradation of N-end rule substrates such as Arg-e^K-βgal and Arg-e^K-DHFRha requires (and is preceded by) the formation of a multi-Ub chain linked to one of two lysines (Lys-15 or Lys-17) in the ~40-residue extension (e^K) at the N terminus of Arg-e^K-DHFRha.

5) Arg-Δe-DHFRha, which lacks the e^K extension, is also degraded (at a lower rate than Arg-e^K-DHFRha) by the N-end rule pathway in reticulocyte extract, and this degradation is also inhibited by MTX (Fig. 3*B*). Only traces of multiubiquitinated Arg-Δe-DHFRha derivatives were observed during its degradation by the N-end rule pathway, and the relative content of these derivatives was further decreased in the presence of MTX (Fig. 2*E* and data not shown).

The extensive ubiquitination of Arg-e^K-DHFRha prior to its degradation indicates that ubiquitination is not rate-limiting for the degradation of Arg-e^K-DHFRha in reticulocyte extract. By contrast, the scarcity of ubiquitinated derivatives of Arg-Δe-DHFRha suggests that ubiquitination of Arg-Δe-DHFRha is among the slowest steps that precede its degradation by the N-end rule pathway. A likely explanation for these differences between Arg-e^K-DHFRha and Arg-Δe-DHFRha is the difference in locations of multi-Ub chains attached to these substrates: in Arg-e^K-DHFRha, a multi-Ub chain is linked to one of the sterically accessible lysines (Lys-15 or Lys-17) in a flexible N-terminal extension (e^K) located outside of the folded DHFR globule (Fig. 1) (Bachmair and Varshavsky, 1989). By contrast, in the case of Arg-Δe-DHFRha a multi-Ub chain must form on one of the numerous but relatively immobile and (presumably) unfavorably located lysines of the folded DHFR globule (Dohmen *et al.*, 1994), thus accounting for both the lower overall rate of Arg-Δe-DHFRha degradation and the low steady-state content of its ubiquitinated derivatives. This interpretation also accounts for the observed difference in the effect of MTX on the relative abundance of multiubiquitinated Arg-e^K-DHFRha and Arg-Δe-DHFRha. Indeed, a block to Arg-e^K-DHFRha degradation due to a conformational stabilization of its DHFR moiety by MTX would not be expected to perturb the formation of a multi-Ub chain linked to the e^K extension of DHFR, as observed. By contrast, a multi-Ub chain linked to Arg-Δe-DHFRha has to form on a lysine of the folded DHFR moiety. This is a slow step, whose rate is further decreased in the presence of MTX, which stabilizes the conformation of DHFR (Matthews *et al.*, 1985; Eilers and Schatz, 1988). (It is assumed that the rate of formation of a multi-Ub chain linked to Arg-Δe-DHFRha is limited by the rate of chain initiation at one of the lysines of DHFR.)

A model that accounts for the findings of this work and is consistent with other evidence is shown in Fig. 4. A major assumption is that the initiation of processive degradation of an N-end rule substrate such as Arg-e^K-DHFRha requires at least a local conformational perturbation of the folded DHFR moiety which can be "utilized" by the proteasome. The probability of this perturbation would be decreased in the presence of MTX, which stabilizes the folded conformation of DHFR. Since MTX inhibits the degradation but not multiubiquitination of Arg-e^K-DHFRha, the relevant conformational perturbation must be a step that occurs after but not before the ubiquitination step (Fig. 4). It is not specified whether this "sufficient" perturbation of DHFR is a thermally driven fluctuation or is at least in part the result of DHFR interactions with components of the N-end rule pathway, including a DHFR-linked multi-Ub chain; the model is consistent with either possibility. Another assumption of the model is that an interaction between a ubiquitinated Arg-e^K-DHFRha substrate and the proteasome-based proteolytic machine is reversible, in that a substrate-proteasome complex dissociates (or enters a state that cannot result in proteolysis; see below) after a stochastically determined time interval (Fig. 4).

Given these assumptions, the near cessation of degradation of DHFR-based N-end rule substrates in the presence of MTX is explained as follows. In the absence of MTX, the mean time interval between the formation of a substrate-proteasome complex and a relevant conformational perturbation of DHFR within the complex is either close to or shorter than the mean lifetime of the complex. In the presence of MTX, its binding to DHFR (and the resulting conformational stabilization of the DHFR globule) decreases the probability of a relevant perturbation of DHFR within the substrate-proteasome complex but does not influence the mean lifetime of the complex (Fig. 4). Specifically, the time interval before perturbation of the substrate is postulated to become significantly longer than the time interval before dissociation of the substrate-proteasome complex, resulting in abortive cycles of targeting and multiubiquitination but little net degradation of a DHFR-based

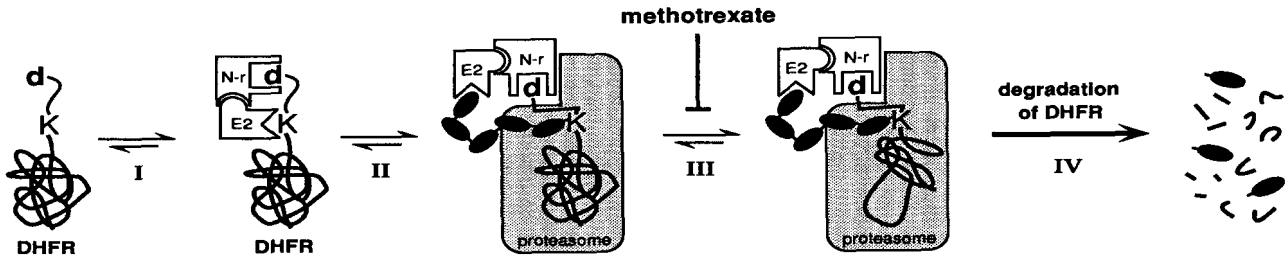

FIG. 4. **On the mechanism of MTX effect.** Each of the four depicted transitions (*I–IV*) in the N-end rule pathway is actually a multistep reaction (Varshavsky, 1992). The shapes of structures are arbitrary. Among the protein-protein complexes shown in this diagram, the interactions between N-recognin (*N-r*) and the Ub-conjugating enzyme (*E2*), and between N-recognin and a DHFR-based N-end rule substrate were demonstrated directly (Bartel *et al.*, 1990; Madura *et al.*, 1993). By contrast, a direct interaction between N-recognin and proteasome is a conjecture that remains to be verified. The path of a multi-Ub chain (*black ovals*) in a complex between proteasome and a multiubiquitinated substrate is unknown. However, if the *E2* enzyme that initiates the formation of a substrate-linked multi-Ub chain is also responsible for the chain elongation, the growing tip of the chain must be in proximity to this *E2*, as shown in the diagram. Superimposition of the substrate and proteasome denotes a complex (of unknown structure) between them. A DHFR-based N-end rule substrate such as Arg-e^K-DHFRha (Fig. 1), bearing a destabilizing N-terminal residue (*d*) and a mobile lysine residue (*K*) outside of the folded DHFR globule, is bound by a complex of N-recognin and *E2* (*step I*). Formation of a lysine-linked multi-Ub chain (the chain's length is arbitrarily set at five Ub moieties) and the binding of a multiubiquitinated substrate by the proteasome take place at *step II*. *In vitro*, the multiubiquitination of an N-end rule substrate can occur in the absence of proteasome; however, it is possible that the binding of a targeted substrate by the proteasome accompanies or even precedes the substrate's multiubiquitination *in vivo*. At *step III*, a local or a global conformational perturbation of DHFR in a complex with the proteasome occurs, resulting in proteolysis (the irreversible *step IV*), which yields short fragments of DHFR and regenerates Ub from a multi-Ub chain. Formation of the MTX·DHFR complex stabilizes the folded DHFR conformation, decreasing the probability of a conformational perturbation of DHFR (*step III*) which can be utilized by the proteasome and thereby inhibiting the degradation of a DHFR-containing substrate. In the case of a protein such as Arg-Δe-DHFRha (Fig. 1), which lacks a targetable lysine residue outside of the DHFR globule, the binding of MTX and the resulting conformational stabilization of DHFR suppress both ubiquitination and degradation of Arg-Δe-DHFRha. See "Materials and Methods" for further details.

N-end rule substrate, as observed.

The understanding of proteasome mechanics is still rudimentary, and therefore the model of Fig. 4 is vague about details of the MTX effect. To cite just one example, the postulated "dissociation" of a substrate-proteasome complex may actually be less than a complete dissociation: for the model to be relevant, it is sufficient that the complex can enter a state in which the proteasome becomes unable to initiate the degradation of a DHFR-based substrate upon an otherwise sufficient perturbation of DHFR.

The degradation of DHFR-based N-end rule substrates *in vivo* (in *S. cerevisiae* cells) was found to be at most weakly inhibited by the addition of MTX to the growth medium.[2] However, in contrast to the above *in vitro* assays (Figs. 2 and 3), in which the N-end rule pathway targets a previously synthesized, folded DHFR, in the *in vivo* assays the binding of MTX to a nascent DHFR-based substrate takes place in kinetic competition with the targeting of the same substrate by the N-end rule pathway. Moreover, while the binding of MTX requires the folded DHFR conformation, the targeting by the N-end rule pathway is expected to occur at any time after the (cotranslational) formation of a destabilizing residue at the N terminus of a substrate. A strategy in which the N-end rule pathway is repressed at first (in a specially constructed yeast strain) and then induced should eliminate the above kinetic competition and allow the effects of MTX to be tested *in vivo* under conditions in which a DHFR-based substrate is folded and MTX-bound before it is targeted by the N-end rule pathway. These experiments are in progress. Interestingly, the *in vivo* degradation of a DHFR-based N-end rule substrate whose DHFR moiety was fused to an unrelated protein such as *S. cerevisiae* Cdc28p (Dohmen *et al.*, 1994) could be inhibited by MTX, in contrast to the degradation of an otherwise identical substrate lacking the Cdc28p domain.[2] The role of a DHFR-linked protein in conferring the sensitivity to inhibition by MTX remains to be understood.

The ability to suppress the *in vivo* degradation of a short lived protein with its cell-penetrating, low M_r ligand would have a number of applications, including the possibility of constructing new types of conditional mutants (Dohmen *et al.*, 1994).

[2] J. Johnston and A. Varshavsky, unpublished data.

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